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Cell surface aminopeptidase A and N activities in human glomerular epithelial cells

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Cell surface aminopeptidase A and N activities in human glomerular epithelial cells. Cell surface aminopeptidases N (APN) and A (APA) have been characterized on cultured human glomerular epithelial cells and a SV40-transformed cell line derived from them. APN had a wide substrate specificity whereas APA only attacked peptides with an acidic N terminal amino acid. Both enzymes also differed by their sensitivity to divalent cations and to aminopeptidase inhibitors. Phorbolmyristate acetate (PMA) stimulated APN but not APA expression after a lag time of 12 hours. An increase of twice the basal value was observed with 10 ng · ml^{-1} PMA. This effect was confirmed by immunofluorescence staining using a specific anti-APN monoclonal antibody. Both ecto- and total enzyme activities were stimulated by PMA. The effect of PMA was suppressed by H7, a PKC inhibitor, and cycloheximide, an inhibitor of protein synthesis. Thrombin (1 to 2.5 U · ml^{-1}) and interferon (IFN)-γ (100 U · ml^{-1}) also stimulated APN activity, the latter after longer exposure of the cells. APA activity was increased by 8-bromo-cAMP and two cAMP-stimulating agents, forskolin and isobutylmethylxanthine (IBMX). A twofold increase above basal value was obtained with 100 μM forskolin after 72 hours of treatment. cAMP-stimulated APA activity was suppressed by cycloheximide. Dexamethasone also stimulated APA activity. The effects of forskolin and dexamethasone were additive. These results demonstrate that APN and APA in glomerular epithelial cells are under different regulations: mitogens and IFN-γ for APN, cAMP and glucocorticoids for APA. This selective expression may imply possible functional consequences in glomerular diseases.

Cell surface peptidases play an important role in signal transduction and communication between cells. Aminopeptidases have recently attracted much interest and four different types of these enzymes, A, N, P and W, have been described [1]. Aminopeptidase N (APN; α-aminoacylpeptide hydrolase, EC 3.4.11.2) acts with a relatively broad substrate specificity on peptides with an N-terminal neutral amino acid. The complete amino acid sequences of human intestinal and rat kidney APN have been deduced from the corresponding cloned cDNA [2, 3]. Sequence comparisons showed that APN was identical to CD 13 (gp 150), a cell surface glycoprotein originally identified on subsets of normal and malignant human myeloid cells [4]. APN is widely distributed with the highest density in the renal cortex and intestinal microvilli [5]. Histochemical studies failed to detect any APN activity in human and murine glomeruli, in contrast with a high activity in the brush border of proximal tubules [6, 7]. However, using more sensitive immunocytochemical techniques, APN was found to be expressed both in glomeruli and proximal tubules on mouse renal sections [8]. Aminopeptidase A or angiotensinase A (APA; L-α-aspartyl or L-α-glutamylpeptide hydrolase, EC 3.4.11.7) acts on peptides with an N-terminal acidic amino acid. In particular, it splits off N-terminal aspartic acid from angiotensin II (Ang II), reducing markedly the activity of this hormone. APA is identical to the murine B-cell differentiation antigen, BP-1/6C3, which is expressed on immature B-lineage cells [9]. High APA activity has been found in human and rat glomeruli, particularly in the podocytes [6, 10]. There is also a positive APA reaction in the brush border of the proximal tubule [6, 7].

Efforts to relate the structure of previously-described cell surface antigens to that of aminopeptidases have allowed the functions of these enzymes to be better appreciated. It has been shown that mouse thymocyte-activating molecule (THAM) was associated with APN and that a monoclonal antibody to this developmentally regulated T cell antigen-activated mouse T lymphocytes [8, 11]. Moreover, interleukin-7 (IL-7) preferentially induced BP-1/6C3 (APA) expression on pre-B cells together with their proliferation [12]. Both findings reinforce the hypothesis of a role for APN and APA in signal transduction.

The aim of the present study was to characterize APN and APA in cultured human glomerular epithelial cells and to study the regulation of their expression in response to mitogens, cytokines, cyclic nucleotides and hormones. Evidence is provided that human glomerular epithelial cells normally possess at their surface both aminopeptidases. Interestingly, control of their expression is different. APN is sensitive to mitogens and interferon (IFN)-γ whereas APA is sensitive to cyclic adenosine monophosphate (cAMP) and glucocorticoids.

Methods

Materials

Materials were obtained from the following suppliers: Dulbecco's modified Eagles medium (DMEM), Ham's F-12 medium, RPMI 1640 medium and 0.05% trypsin-0.02% EDTA from Flow Laboratories (Irvine, Ayrshire, UK); fetal calf serum (FCS), penicillin G and streptomycin sulphate from

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Gibco (Grand Island, New York, USA); anti-myosin and anti-urokinase antisera from Miles (Paris, France); L-glutamyl p-nitroanilide from Serva (Heidelberg, Germany); L-amino acid p-nitroanilide substrates of aminopeptidase N, amastatin, bestatin, 1,10-phenanthroline, *E. coli* 026 B6 lipopolysaccharide, collagenase, 8-bromo-cyclic AMP, 8-bromo-cyclic GMP, calcium ionophore A23187, phorbol-12-myristate-13-acetate (PMA), actinomycin D, cycloheximide and HEPES from Sigma (St. Louis, Missouri, USA); angiotensin II (Hypertensin) from Ciba-Geigy (Basel, Switzerland); human recombinant interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ from Genzyme corporation (Cambridge, Massachusetts, USA); thiorphan from Bioprojet (Paris, France); 1(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) from Seikagaku (Tokyo, Japan). Anti-von Willebrand factor, a pool of monoclonal antibodies allowing this high molecular mass protein to be adequately detected, was a gift from Pr. D. Meyer (Paris, France). All other chemicals were of reagent grade and were used without further purification. Stock solutions of PMA in dimethylsulphoxide (DMSO) were prepared (1 mg · ml^{-1}) and were kept at −20°C. Plastic flasks and dishes were obtained from Nunc (Roskilde, Danemark).

Cell culture

Human glomerular epithelial cells were prepared as previously described [13]. Renal cortical tissue was obtained from human cadaver kidneys judged to be unsuitable for transplantation. Glomeruli were isolated by successive passages through different sieves. Human glomerular epithelial cells were obtained by collagenase digestion of isolated glomeruli. These cells were cultured in RPMI 1640 medium buffered with 20 mM HEPES to pH 7.4 and supplemented with 2 mM glutamine, 10% fetal calf serum, 100 U · ml^{-1} penicillin and 100 μg · ml^{-1} streptomycin. They were maintained at 37°C under a humidified atmosphere of 5% CO_2/95% air. Human glomerular epithelial cells reached confluence after 10 to 15 days. They were subcultured and studied after one to three passages. Human glomerular epithelial cells were identified by immunofluorescence techniques. Staining was positive with antisera directed against urokinase and neutral endopeptidase, respectively. No staining was obtained with anti-myosin and anti-von Willebrand factor antisera.

Most studies were also carried out on a SV40-transformed human podocyte cell line that derives from a primary culture of podocytes obtained from a pure preparation of unencapsulated glomeruli which has been transfected with a replication-defective SV-40 plasmid [14]. The resulting cell line after 12 passages was grown in a hormonally-defined medium [DMEM-Ham's F12 1:1 (vol/vol); glutamine, 2 mM; sodium selenate, 30 nM; transferin, 5 μg · ml^{-1}; insulin, 5 μg · ml^{-1}; dexamethasone, 50 nM; HEPES, 20 mM, pH 7.4] containing 2% fetal calf serum. Clone A4 used in this study exhibits the major characteristics of podocytes, including expression of vimentin, podocalyxin, neutral endopeptidase, heparan sulfate proteoglycans, and production of type IV collagen [14].

Immunofluorescence studies

Expression of APN was detected using a specific monoclonal antibody (code number 312 A 27) raised in the laboratory [15]. For demonstrating cell surface APN, cells were fixed in 4% paraformaldehyde during 15 minutes at room temperature. Other cells were treated with methanol to allow the antibody to penetrate the cells. Cells were then washed three times successively in 0.1 M glycine and phosphate-buffered saline. APN was demonstrated after incubation with 100 μg · ml^{-1} anti-APN antibody for 30 minutes at room temperature followed by a second incubation with fluorescent anti-mouse IgG also during 30 minutes, and by three washings in phosphate-buffered saline. Parallel studies could not be performed for APA detection due to the lack of specific antibody against human APA.

Enzyme activities

Cell surface APN activity was determined on confluent monolayers. This technique compared with the study of cell suspensions might underestimate the total ectoenzyme activity since only a part of the cells can gain contact with the substrate. It has, however, the advantage of being able to study cells at rest maintained under more physiological conditions. Cells in 24-well plates were rinsed three times and incubated at 37°C in calcium-free phosphate-buffered saline, pH 7.4, supplemented with 1 mM $MgCl_2$. In some experiments, 50 mM HEPES buffer, pH 7.4, containing 130 mM NaCl and 1 mM $MgCl_2$ was used. Enzyme activity was determined under zero-order kinetic conditions, using 1.5 mM alanine p-nitroanilide as a substrate unless otherwise indicated. After an incubation of 5 to 20 minutes at 37°C, the amount of p-nitroanilide formed was measured in the supernatant. It was determined from a standard curve by reading at an OD of 405 nm using a Perkin-Elmer double beam spectrophotometer. Cell-free and substrate-free blanks were run in parallel.

Cell surface APA activity was determined from the hydrolysis of 10 mM α-glutamyl-p-nitroanilide over 10 to 30 minutes by cells incubated in 50 mM Tris-HCl buffer, pH 8.0, containing 130 mM NaCl and 10 mM $CaCl_2$.

Total enzyme activities for both enzymes were measured in cells which had been sonicated using a Branson sonifier (model W 185 D, Branson Sonic Power Co, Danbury, Connecticut USA) at position 3. The tube containing the cells was immersed in an ice-water bath and the sample was sonicated for 60 seconds.

Enzyme activities were expressed as nmol p-nitroanilide formed per minute and per mg of cell protein.

K_m and V_{max} determinations

Initial reaction velocities (V) were determined over substrate concentration (S) ranges of 0.1 to 5 mM and 0.25 to 10 mM for APN and APA, respectively. Data were plotted according to Hofstee (V vs. V/S) to determine the K_m and V_{max} values [16].

Cell protein

After appropriate digestion with 1 M NaOH, cell protein was determined by the method of Lowry et al [17] using bovine serum albumin as the standard.

Statistical analysis

Results are expressed as means ± SE. Comparisons between groups were made using Student's *t*-test for unpaired values and analysis of variance. Correlations between two parameters were estimated by regression analysis.

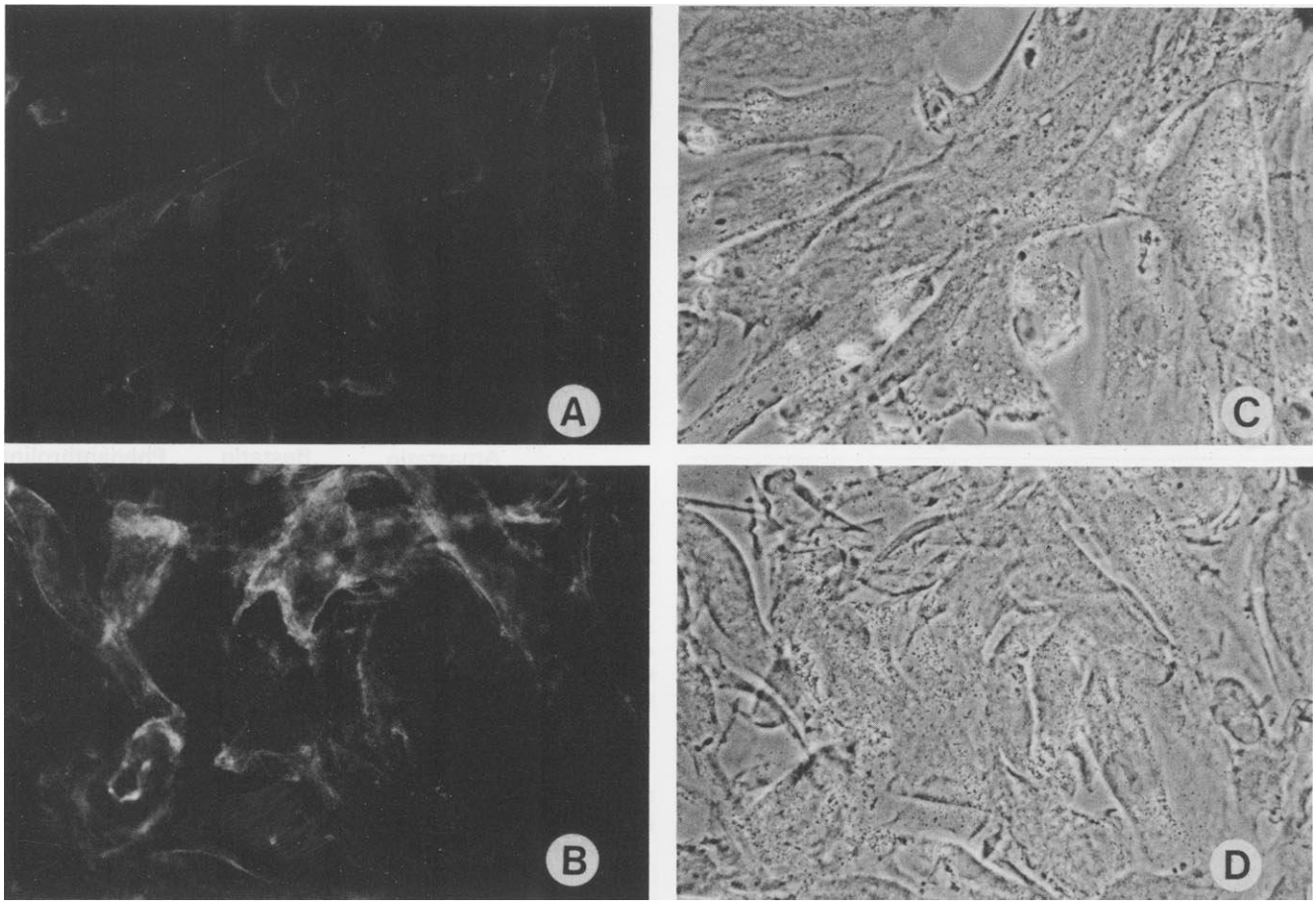

Fig. 1. *Expression of aminopeptidase N in human glomerular epithelial cells.* The enzyme was detected by immunofluorescence using a specific monoclonal antibody. Cells were fixed with 4% paraformaldehyde before incubation with the antibody under control conditions **(A)** or after exposure to 10 ng · ml^{-1} PMA for 48 hours **(B)**. Phase contrast micrographs of the same fields are also shown **(C, D)**. Films for immunofluorescence pictures (ILFORD HP5 plus, 400 ASA) were exposed for 18 seconds to the illuminated microscope field and the resulting negatives were printed under the same conditions. Note increased intensity of fluorescence in PMA-treated cells (B).

Results

Cell surface location of APA and APN

Human glomerular epithelial cells were found to hydrolyze substrates of both APA and APN. Enzyme activity was determined with intact cells. Cell viability measured by the trypan blue exclusion method was preserved during the incubation time. The proportion of intact cells exceeded 97% after enzyme determination and was not different from that found in control preparations. The following is taken as evidence that both enzyme activities were present at the cell surface: (1) Substrates were hydrolyzed by intact cultured cells and their respective products were released in the extracellular medium; (2) The amount of substrate hydrolyzed over 5 to 30 minutes was linearly related to time, making it unlikely that a significant fraction of substrate had been hydrolyzed after entering the cells; (3) Immunofluorescence studies after fixation with paraformaldehyde, a fixative that only exposes antigens expressed at the cell surface, showed a marked staining with a monoclonal anti-APN antibody (Fig. 1). Methanol pretreatment of the cells increased the staining of epithelial cells obtained with this anti-APN antibody; (4) APA and APN activities were absent in the incubation and the culture medium, indicating that the enzymes were not released from the cells. Substantial portions of enzyme activities were expressed at the cell surface of epithelial cells with some variability according to the preparation ($1.17 \pm 0.06 - 3.74 \pm 0.17$ nmol · min^{-1} · mg^{-1} for APA and $1.42 \pm 0.09 - 3.16 \pm 0.22$ nmol · min^{-1} · mg^{-1} for APN). Total enzyme activities measured in sonicated cells were greater ($2.47 \pm 0.10 - 5.92 \pm 0.15$ nmol · min^{-1} · mg^{-1} for APA and $2.50 \pm 0.11 - 6.51 \pm 0.37$ nmol · min^{-1} · mg^{-1} for APN) showing that both enzymes were also present within the cells.

Characteristics of APA and APN

APN was shown to have a wide substrate specificity (Table 1). Substrates were hydrolyzed in the following order of reactivity: alanine p-nitroanilide > leucine p-nitroanilide > lysine p-nitroanilide > arginine p-nitroanilide > glycine and valine p-nitroanilide. APA activity was only studied with glutamic acid-α-p-nitroanilide.

The pH versus activity profile of human glomerular epithelial cell APN was studied with two different buffers: phosphate-buffered saline and HEPES buffer. The optimal pH was 7.4 with

Table 1. Substrate specificity of surface aminopeptidases in human glomerular epithelial cells

Substrate	Amniopeptidase activity $nmol \cdot mg^{-1} \cdot min^{-1}$
Alanine p-NA	2.51 ± 0.06
Leucine p-NA	2.17 ± 0.11
Lysine p-NA	1.06 ± 0.05
Arginine p-NA	0.76 ± 0.02
Glycine p-NA	0.21 ± 0.01
Valine p-NA	0.19 ± 0.005
Glutamic acid p-NA	2.27 ± 0.12

Cells were incubated for 20 minutes with 1 mM of substrate. Values are means ± SE of 4 determinations.

Table 2. Effect of divalent cations on surface aminopeptidase N (APN) and A (APA) activities of human glomerular epithelial cells

Divalent cation	APN	APA
	$nmol \cdot min^{-1} \cdot mg^{-1}$	
None	2.95 ± 0.08	0.94 ± 0.03
EDTA, 5 mM	2.52 ± 0.04	0.41 ± 0.02
Ca^{2+}, 1 mM	3.29 ± 0.12	1.21 ± 0.08
Ba^{2+}, 1 mM	3.22 ± 0.39	1.20 ± 0.04
Mg^{2+}, 1 mM	2.97 ± 0.27	0.90 ± 0.05
Mn^{2+}, 1 mM	2.56 ± 0.22	1.14 ± 0.06

Cells were incubated for 20 minutes in 50 mM HEPES buffer, pH 7.4, for aminopeptidase N or for 30 minutes in 50 mM Tris-HCl buffer, pH 8.0 for aminopeptidase A with EDTA or the divalent cation selected. Values are means ± SE of 4 determinations.

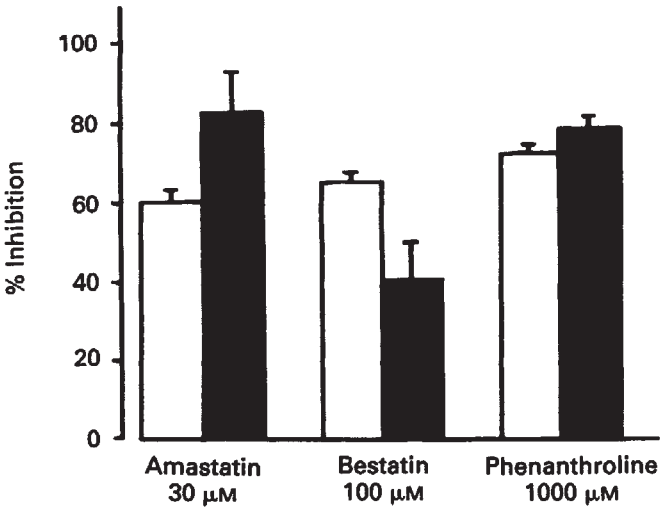

Fig. 2. *Comparative effects of three different inhibitors on surface aminopeptidase A and N activities in human glomerular epithelial cells.* Percent of inhibition is shown as mean ± SE of 4 determinations. Data were analyzed using Student's *t*-test. Activities of both enzymes were significantly reduced ($P < 0.01$) in comparison with control after exposure to each of the inhibitors studied. Control values were 1.88 ± 0.10 and 1.26 ± 0.06 nmol · min^{-1} · mg^{-1} for aminopeptidases N and A, respectively.

HEPES. It ranged between 7.4 and 8.0 in phosphate-buffered saline. APA activity was determined in 50 mM Tris-buffer. An optimal pH was observed at 7.8 to 8.4. An identical pH profile with SV-40 transformed human epithelial cells was demonstrated for both APN and APA activities.

The effect of divalent cations on surface APA and APN activities is presented in Table 2. Quite similar APA activities were obtained with calcium, barium and manganese at 1 mM concentration. Activity was lower in the presence of 1 mM magnesium and was markedly inhibited by 5 mM EDTA. APN activity was apparently the same in the presence of 1 mM calcium, barium or magnesium. The substantial residual activity of APN found in the absence of divalent cations, or in the presence of 5 mM EDTA is probably due to the presence of zinc normally bound to the residues participating in the catalytic reaction [3].

Kinetic studies were performed with L-glutamic acid-α-p-nitroanilide (0.25 to 10 mM) for APA activity, and with L-alanine p-nitroanilide (0.1 to 5 mM) for APN activity. The data were transformed according to Hofstee. Regression analysis provided apparent K_m and V_{max} values of 0.48 mM and 3.82 nmol · min^{-1} · mg^{-1}, respectively, for APA and of 0.70 mM and 4.4 nmol · min^{-1} · mg^{-1}, respectively, for APN. With SV-40 transformed epithelial cells apparent K_m and V_{max} values of 0.29 mM and 1.52 nmol · min^{-1} · mg^{-1}, respectively, for APA and of 1.01 mM and 2.10 nmol · min^{-1} · mg^{-1}, respectively, for APN were obtained. APA activity was inhibited by 43.1% with 1 mM angiotensin II (Hypertensin). This effect was observed keeping substrate concentration of glutamyl p-nitroanalide at 1.5 mM. Inhibition of APA activity was also observed with acidic amino acids. Aspartic and glutamic acid (10 mM) inhibited APA activity by 20.2% and 15.2%, respectively.

Effect of aminopeptidase inhibitors

Three known inhibitors of aminopeptidases, amastatin, bestatin and 1,10-phenanthroline were used (Fig. 2). 1,10-phenanthroline, a chelating agent, was the least potent inhibitor. Used at a concentration of 1 mM, it had approximately the same inhibitory effect on APA (78.6%) and APN (71.3%) activities. Amastatin (30 μM) produced a significantly higher inhibition of APA (82.5%) than of APN (60%). In contrast, bestatin (100 μM) was more inhibitory for APN (64.9%) than for APA (39.7%).

Induction of surface aminopeptidases

Effect of phorbolmyristate acetate (PMA), thrombin and cytokines. Glomerular epithelial cells were treated for 48 hours with A 23187 calcium ionophore (1 μM), IL-1β (25 U · ml^{-1}), TNFα (25 ng · ml^{-1}), *E. coli* lipopolysaccharide (10 μg · ml^{-1}) and PMA (10 ng · ml^{-1}), a tumor promoter and maturation-inducing agent (Table 3). There was a statistically significant increase in APN activity with IL-1β ($P < 0.01$), LPS ($P < 0.01$) and PMA ($P < 0.001$). Since IL-1β could have been degraded during the period of incubation due to the presence of neutral endopeptidase (enkephalinase, EC 3.4.24.11) at the surface of glomerular epithelial cells [18], parallel experiments were performed in the presence of thiorphan (10 μM), an inhibitor of neutral endopeptidase. The increase in APN activity above basal value after treatment of the cells by 25 U · ml^{-1} IL-1β was similar with (3.70 ± 0.57 nmol · min^{-1} · mg^{-1}) and without (3.55 ± 0.27 nmol · min^{-1} · mg^{-1}; $P > 0.05$) thiorphan. Combined treatments by PMA and IL-1β, or PMA and LPS produced additive increases of APN activity. TNFα was without effect on APN activity at the concentration tested. APA activity

Table 3. Effect of various agents on human glomerular epithelial cell aminopeptidases A (APA) and N (APN)

Agent	APN	APA
	nmol · min⁻¹ · mg⁻¹	
None	2.84 ± 0.05	0.85 ± 0.03
A 23187 Ca^{2+} ionophore, 1 μM	2.34 ± 0.03	0.92 ± 0.06
IFN-γ, 100 U · ml⁻¹	3.09 ± 0.08	0.82 ± 0.04
PMA, 10 ng · ml⁻¹	4.69 ± 0.10[b]	0.76 ± 0.02
TNFα, 25 ng · ml⁻¹	2.89 ± 0.05	0.82 ± 0.02
PMA, 10 ng · ml⁻¹ + TNFα, 25 ng · ml⁻¹	4.71 ± 0.21[b]	0.76 ± 0.01
IL-1β, 25 U · ml⁻¹	3.20 ± 0.12[a]	0.80 ± 0.02
PMA, 10 ng · ml⁻¹ + IL-1β, 25 U · ml⁻¹	5.70 ± 0.17[b]	0.80 ± 0.06
LPS, 10 μg · ml⁻¹	3.38 ± 0.05[a]	0.91 ± 0.07
PMA, 10 ng · ml⁻¹ + LPS, 10 μg · ml⁻¹	5.30 ± 0.04[b]	0.86 ± 0.05

Cells were cultured with agents in the concentrations stated for 48 hours. Values are means ± SE of 4 to 10 determinations. Data were analyzed using Student's *t*-test or two way analysis of variance when combined effects were studied. The effects of LPS and IL-1β were additive to those of PMA (no significant interaction).

[a] $P < 0.01$ and [b] $P < 0.001$ vs. control

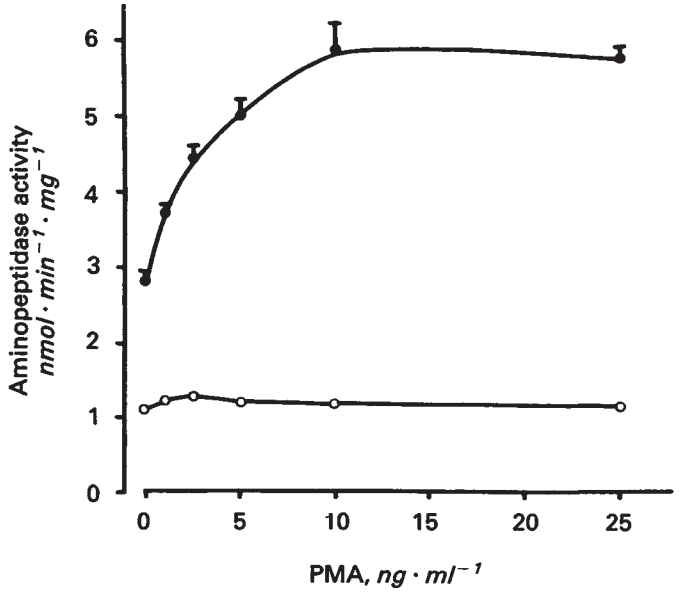

Fig. 3. *Aminopeptidase N (closed circles) and aminopeptidase A (open circles) activities plotted against phorbolmyristate acetate (PMA) concentration.* Human glomerular epithelial cells were cultured with PMA for 48 hours. Means ± SE of 4 determinations are shown. Data were analyzed using Student's *t*-test. Aminopeptidase N activity at any concentration of PMA studied was significantly greater than control ($P < 0.01$). There was no significant change of aminopeptidase A activity.

was not changed by any of these products. We more thoroughly studied the effect of PMA since this agent was the most potent on APN of those used. Stimulation by PMA was apparent only after 12 hours of incubation and reached a maximum at 48 to 72 hours. The dose-response curve at 48 hours is shown in Figure 3. PMA at 1 ng · ml⁻¹ produced a significant increase of APN activity. The maximal effect was obtained at 10 ng · ml⁻¹ (16 nM). PMA stimulated both surface and total APN activities. APN activity was 5.92 ± 0.56 and 2.85 ± 0.07 nmol · min⁻¹ · mg⁻¹ for the ectoenzyme, 6.46 ± 0.11 and 3.62 ± 0.06 nmol · min⁻¹ · mg⁻¹ for the total enzyme with and without PMA (10 ng · ml⁻¹), respectively ($P < 0.01$). The stimulatory effect of PMA on APN expression at the surface of glomerular epithelial cells was confirmed by immunofluorescence. Staining in the presence of an anti-APN monoclonal antibody was more marked after exposure of the cells to 10 ng · ml⁻¹ PMA for 48 hours than under control conditions (Fig. 1). To verify whether the effect of PMA was mediated by protein kinase C (PKC) and whether it required a new protein synthesis, human glomerular epithelial cells in culture were pretreated with H7, an inhibitor of PKC, or with cycloheximide, an inhibitor of protein synthesis (Table 4). When added to human glomerular epithelial cells one hour before PMA, H7 prevented APN induction by PMA. In addition, PMA did not induce APN activity of cycloheximide-pretreated epithelial cells.

Table 4. Effect of protein kinase C and protein synthesis inhibition on PMA-induced aminopeptidase N of human glomerular epithelial cells

Agent	Aminopeptidase N activity *nmol · min⁻¹ · mg⁻¹*
Control	1.93 ± 0.12
PMA, 10 ng · ml⁻¹	3.15 ± 0.27[a]
H7, 50 μM	2.12 ± 0.15
PMA, 10 ng · ml⁻¹ + H7, 50 μM	2.04 ± 0.19
Cycloheximide, 0.5 μg · ml⁻¹	2.08 ± 0.07
PMA, 10 ng · ml⁻¹ + cycloheximide, 0.5 μg · ml⁻¹	2.15 ± 0.02

Cultured epithelial cells were pretreated for 1 hour with H7 or cycloheximide and then incubated with PMA, 10 ng · ml⁻¹. Values are means ± SE of 4 determinations. Data were analyzed using Student's *t*-test.

[a] $P < 0.001$ vs. control

The fact that stimulation of PKC was necessary for the increased expression of APN led us to study the effect of endogenous activators of PKC. We chose to study thrombin, which is a mitogen for glomerular cells acting via the phospholipase C pathway [19]. Cells were exposed to thrombin in the concentration range of 0.1 to 2.5 U · ml⁻¹ for 48 hours. There was a dose-dependent increase of surface APN activity. Values of 4.23 ± 0.06, 4.93 ± 0.17 and 5.58 ± 0.08 nmol · min⁻¹ · mg⁻¹ were observed under basal conditions and in the presence of 1 and 2.5 U · ml⁻¹ thrombin, respectively, the latter two representing 116.5 ($P < 0.05$) and 132% ($P < 0.001$) increases of basal value.

Although no stimulatory effect on APA and APN activities had been obtained after 48 hours of incubation with IFN-γ, we thought it of interest to study this cytokine over longer periods of incubation since its role in cell differentiation has been demonstrated in other preparations [20]. Cells were cultured with IFN-γ (100 U · ml⁻¹) for up to 11 days. Surface APN activity increased markedly with time. A statistically significant increase was observed from day 5 ($P < 0.05$) with a maximum increase at day 11 (218% above basal value; Fig. 4). APN activity in response to IFN-γ was more stimulated in proliferating than in confluent epithelial cells (data not presented). In contrast, IFN-γ had no significant effect on APA activity.

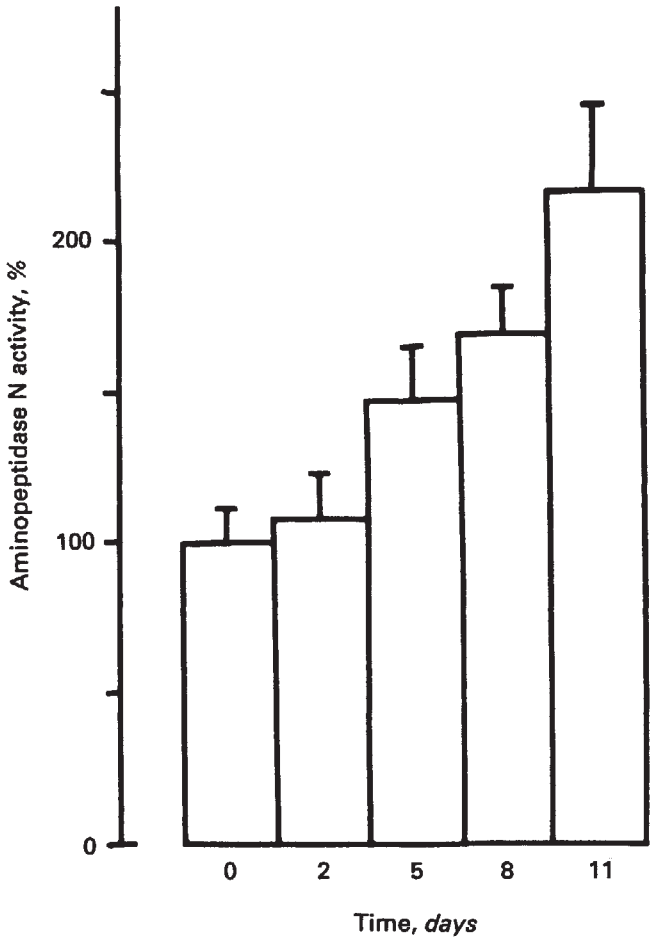

Fig. 4. *Effect of IFN-γ on the expression of aminopeptidase N activity in glomerular epithelial cells.* Cells were cultured with IFN-γ (100 U · ml^{-1}) for up to 11 days. Values are means ± SE of 4 determinations expressed as % of basal activity. Data were analyzed using Student's *t*-test. Aminopeptidase N activity was significantly greater than control ($P < 0.01$) from the fifth day of incubation.

Effect of cyclic nucleotides. Epithelial cell aminopeptidase activity was also modulated by cAMP. Parental and transformed human glomerular epithelial cells were cultured for 72 hours with 8-bromo-cAMP, IBMX, an inhibitor of phosphodiesterase, or forskolin, a diterpene which activates the catalytic moiety of adenylate cyclase. 8-bromo-cAMP produced a dose-dependent and significant increase in APA activity in both normal and transformed epithelial cells. Values 68 and 85% above control were found in the presence of 500 μM 8-bromo-cAMP for these two preparations, respectively (Fig. 5A). In contrast, the increase in APN activity was relatively small, reaching only values 11.8 and 14.2% above control under the same conditions. Treatment of epithelial cells by IBMX produced also a marked increase in APA activity (58 and 65% increases in parental and transformed cells, respectively) and a small increase not exceeding 10% in APN activity (Fig. 5B). The highest increase in APA activity occurred in the presence of forskolin. Treatment by this drug stimulated APA activity in a dose-dependent manner. After a 72 hour treatment by 100 μM forskolin, surface APA increased by 140 and 200% in normal and transformed epithelial cells, respectively (Fig. 5C). At this same concentration, forskolin markedly inhibited epithelial cell proliferation (17.8 ± 0.4 vs. 24.6 ± 2.1 μg cell protein per well) indicating that the increase in enzyme activity was not due to a mitogen effect. APA activity in the presence of 10 μM forskolin was still significantly increased, reaching values 81.3 and 116% above control in normal and transformed cells, respectively. Increases in APN activity under the same conditions did not exceed 16.7 and 22.5% above basal value. Forskolin stimulated both surface and total APA activities (data not presented). Forskolin-dependent increase in APA activity could be prevented by pretreatment with either actinomycin D, an inhibitor of RNA synthesis, or cycloheximide, an inhibitor of protein synthesis (Table 5). In contrast with cAMP and cAMP-stimulating agents, treatment of human glomerular epithelial cells by 8-bromo-cGMP (500 μM) for 72 hours had no significant effect on APA and APN activities.

We recently demonstrated that treatment of human glomerular epithelial cells by dexamethasone increased ecto-APA activity in a dose- and time-dependent manner [21]. Therefore we felt it of interest to study the combined effects of dexamethasone (0.5 μM) and forskolin (10 μM) or 8-bromo-cAMP (500 μM). Surface APA activity was induced significantly by each of these agents studied alone. Dexamethasone and forskolin had additive effects whereas 8-bromo-cAMP potentiated the effect of dexamethasone (Fig. 6). There were small increases in APN activity after a 72 hour treatment by dexamethasone and 8-bromo-cAMP. Both agents in combination had an additive effect. Indeed, APN activity increased by 25.5% after dexamethasone (0.5 μM), 14.4% after 8-bromo-cAMP (500 μM) and 35% with both agents.

Discussion

The present study demonstrates that intact human glomerular epithelial cells in culture hydrolyze synthetic substrates of APA and APN. If the presence of APA in the human podocytes has already been shown, that of APN was unexpected, this enzyme being essentially localized in the human kidney in the brush border of the proximal tubule [6, 10]. We have also recently shown that APN was expressed on human cultured mesangial cells [22]. Whereas substrate specificity is relatively broad for APN suggesting that it can attack a variety of peptides, APA hydrolyzes only peptides with an acidic N terminal residue such as aspartic acid. This is the case of angiotensin II which can be considered as a preferential substrate of the enzyme. We have only studied APA and APN present at the cell surface since we measured enzyme activities on intact cells in monolayer. However, both enzymes are also present in the cytosol of podocytes, as shown by the greater activity per mg of protein found in sonicated cells. The properties of APA and APN that we described in glomerular epithelial cells are close to those previously reported in other preparations. Rat renal APA exhibited a K_m of 0.23 mM, and its activity was competitively inhibited by angiotensin II [23]. APA activity in human cultured endothelial cells and in human placental microvilli was inhibited by phenanthroline [24, 25] and stimulated by calcium chloride [25]. Surface APN exhibited K_m of 0.40 and 0.86 mM in human lymphocytes [26] and human mesangial cells [22], respectively, and its activity was inhibited in both preparations by bestatin,

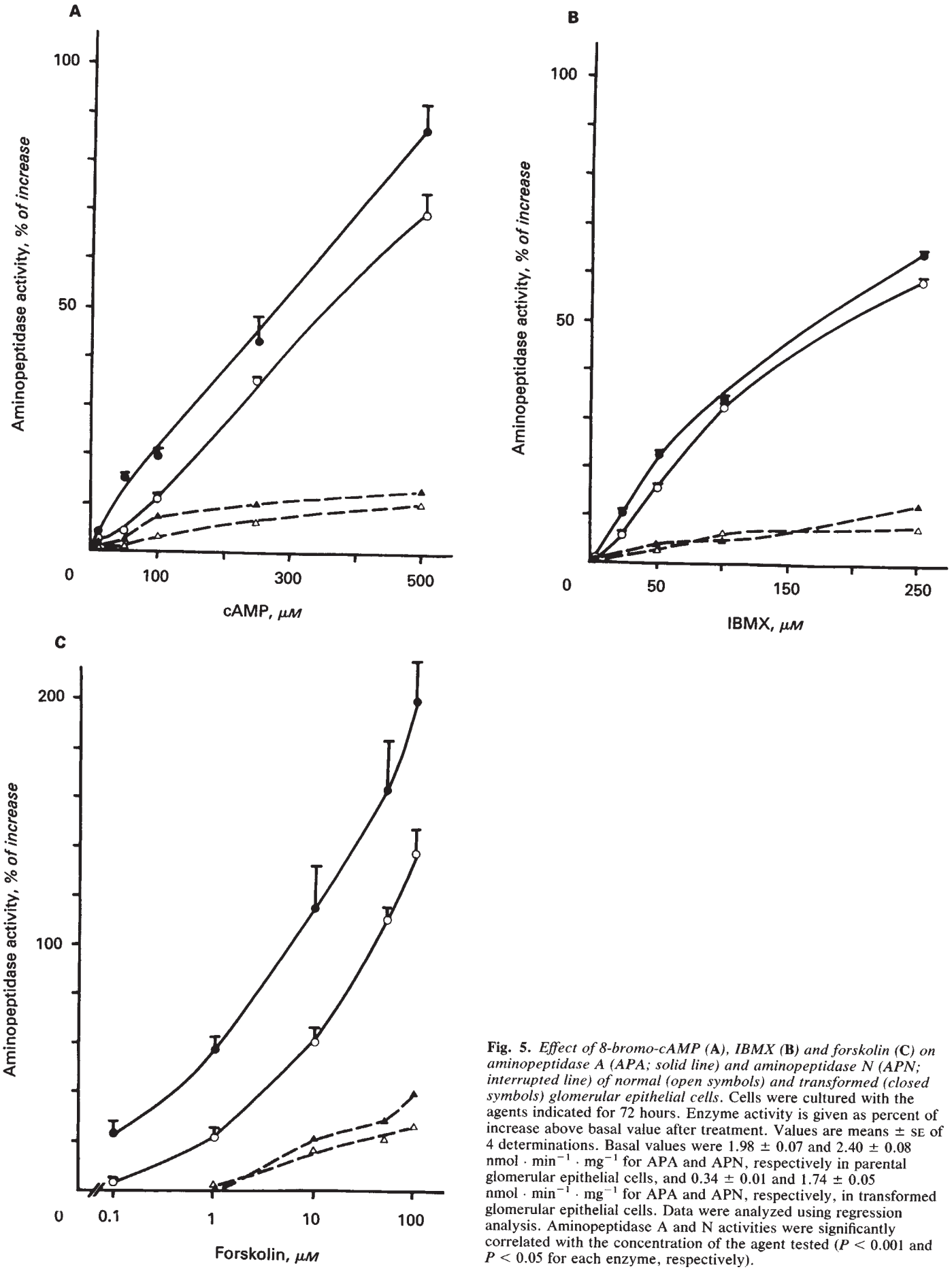

Fig. 5. *Effect of 8-bromo-cAMP* (**A**), *IBMX* (**B**) *and forskolin* (**C**) *on aminopeptidase A (APA; solid line) and aminopeptidase N (APN; interrupted line) of normal (open symbols) and transformed (closed symbols) glomerular epithelial cells.* Cells were cultured with the agents indicated for 72 hours. Enzyme activity is given as percent of increase above basal value after treatment. Values are means ± SE of 4 determinations. Basal values were 1.98 ± 0.07 and 2.40 ± 0.08 nmol · min^{-1} · mg^{-1} for APA and APN, respectively in parental glomerular epithelial cells, and 0.34 ± 0.01 and 1.74 ± 0.05 nmol · min^{-1} · mg^{-1} for APA and APN, respectively, in transformed glomerular epithelial cells. Data were analyzed using regression analysis. Aminopeptidase A and N activities were significantly correlated with the concentration of the agent tested ($P < 0.001$ and $P < 0.05$ for each enzyme, respectively).

Table 5. Effect of inhibition of protein and RNA synthesis on forskolin-induced aminopeptidase A activity in human glomerular epithelial cells

Treatment	Aminopeptidase A activity $nmol \cdot min^{-1} \cdot mg^{-1}$
Control	1.28 ± 0.08
Forskolin, 10 μM	2.41 ± 0.28[a]
Cycloheximide, 0.1 $\mu g \cdot ml^{-1}$	1.04 ± 0.05
Forskolin, 10 μM + cycloheximide, 0.1 $\mu g \cdot ml^{-1}$	1.12 ± 0.03
Actinomycin D, 0.1 $\mu g \cdot ml^{-1}$	1.10 ± 0.05
Forskolin, 10 μg + actinomycin D, 0.1 $\mu g/ml^{-1}$	1.34 ± 0.04

Cells were preincubated with cycloheximide or actinomycin D for 1 hour before addition of forskolin and were then incubated for a further period of 72 hours. Data are means ± SE of 4 determinations. Data were analyzed using Student's *t*-test.

[a] $P < 0.01$ vs. control

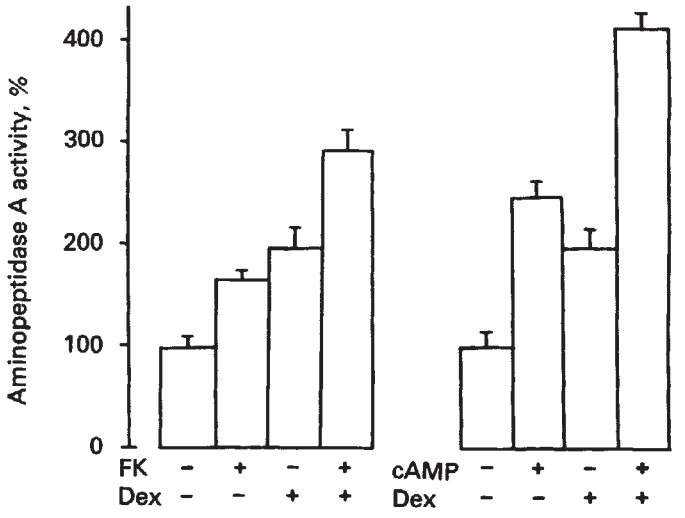

Fig. 6. *Effect of 0.5 μM dexamethasone (Dex), 10 μM forskolin (FK) and 500 μM 8-bromo-cAMP on aminopeptidase A (APA) activity of human glomerular epithelial cells.* Cells were cultured with the agents indicated for 72 hours. Enzyme activity is given as percent of basal value after treatment. Values are means ± SE of 4 determinations. Data were analyzed using Student's *t*-test or two-way analysis of variance when combined effects were studied. The increase of APA activity after dexamethasone, forskolin or cAMP treatment was significant vs. control ($P < 0.001$). The effect of forskolin was additive to that of dexamethasone (no significant interaction). The effect of cAMP plus dexamethasone exceeded the sum of activities obtained with each of these agents separately (significant interaction, $P < 0.025$).

phenanthroline and amastatin [22, 26]. Its K_m in a rat kidney homogenate was 0.24 mM [23].

Surface aminopeptidase A and N activities of human glomerular epithelial cells were found to be under different regulations. PMA, a tumor-promoting phorbol ester, IL-1 and IFN-γ were only active on APN, whereas cAMP, whatever the reason for its accumulation, and dexamethasone, a glucocorticoid hormone, stimulated mainly the activity of APA. The stimulation of APA and APN activities by these different mediators was not the result of their effect on cell growth since the amount of protein per well was either diminished in the case of forskolin or unchanged in the other cases. We previously reported a similar stimulatory effect of PMA on APN of human mesangial cells [22]. The initial step of PMA mode of action is the activation of PKC which results in the induction of c-*fos* and c-*jun*. Both compounds interact with a specific DNA element termed AP-1 binding site, resulting in gene transcription and protein synthesis [27]. Such a mechanism may explain the stimulatory effect of PMA on APN expression since H7, an inhibitor of PKC, and cycloheximide, an inhibitor of protein synthesis, were both suppressive. Moreover, APN stimulation by PMA required a lag time of 12 to 24 hours. Similar effects of phorbolesters on the expression of cell surface proteins have already been reported. For example, tetradecanoyl phorbol acetate (TPA) induced the FcR II type of IgG receptors which is involved in monocyte cytotoxicity and phagocytosis of IgG-coated particles [28]. Phorbolesters also increased the production of enzymes involved in angiogenesis and tumor extension such as collagenase, plasminogen activator and urokinase [29, 30]. IFN-γ is considered to be an important regulating factor in a variety of cells including macrophages, lymphocytes, tumor cells and fibroblasts. It induces the appearance of new surface markers or receptors such as class II MHC antigens and Fc receptors for monomeric IgG (FcR I) [20]. It also induces cells to express ectoenzymes such as 5'-nucleotidase and adenosine deaminase on monocytes and functional activities characteristic of cells differentiating along the monocytic pathway [31]. We demonstrate in the present study that glomerular epithelial cells may also be the target of IFN-γ. This cytokine could alter the phenotype of these cells in a similar manner to PMA as shown by the increased activity of cell surface aminopeptidase N. The same differentiating effect of PMA and IFN-γ has already been observed for 5' nucleotidase in monocytes [31] and Fcγ receptors in a human monoblast cell line [27].

Regulation of APA activity was found to be different from that of APN. We demonstrated previously that dexamethasone increased ecto-APA activity of human glomerular epithelial cells in a dose- and time-dependent manner. Maximum increase of APA activity occurred after treatment by 0.5 μM dexamethasone for five days. Actinomycin D and cycloheximide prevented and RU 38486, a glucocorticoid receptor antagonist, suppressed the dexamethasone-induced increase in APA activity [21]. The stimulatory effect of dexamethasone on cell surface APA activity was confirmed in the present study with an increase of twice basal value after 72 hour exposure to 0.5 μM dexamethasone. In addition to dexamethasone, we found that APA activity was markedly stimulated by cAMP. The most marked increase was observed with forskolin, an ubiquitous stimulator of the catalytic moiety of adenylate cyclase. 8-bromo-cyclic AMP, a cell penetrating analogue of cAMP, also stimulated basal APA activity by 85%. The effect of IBMX, a phosphodiesterase inhibitor, confirmed that cAMP was the common mediator of the three agents tested. The effect of cAMP on APN was significant but of a much lower magnitude, about one tenth of that obtained for APA at the greatest concentrations of the three agents studied. Interestingly, transformed glomerular epithelial cells responded to cAMP similarly to their parental cells. This confirms that the transformed cell line possesses the characteristics of the normal cells from which it derives [14] and could thus represent an abundant and stable material for their study. cAMP effect needed a lag time of 24

hours and was maximum after 72 hours of incubation. It was also inhibited by cycloheximide and actinomycin, confirming that its mode of action involved protein synthesis. Such an effect of cAMP on enzyme expression has been extensively described in many systems including 5′-nucleotidase in rat mesangial cells [32], ecto Ca^{2+} ATPase in an hepatoma cell line [33], and γ glutamyl transpeptidase in LLC-PK1 cells derived from the pig renal proximal tubule [34]. The relatively slow rate of induction of transcription by cAMP and the sensitivity to cycloheximide suggest that the gene of APA belongs to the slow responding class of the cAMP-regulated genes [34]. It differs from the genes of a variety of other enzymes whose transcription depends on the rapid activation by the complex cAMP-protein kinase of the cAMP regulatory element (CRE) binding protein or the AP-2 element binding protein. The effect of cAMP and dexamethasone were additive, suggesting that these two agents do not act along the same pathway.

Polarized membrane expression of brush border hydrolases in primary cultures of kidney proximal tubular cells depends on cell differentiation. APN, neutral endoproteinase and dipeptidyl-peptidase IV were shown to be distributed essentially at the surface of these cells after incubation with dexamethasone, whereas they were abnormally concentrated in a cytoplasmic vesicle compartment in the presence of serum or in the absence of dexamethasone [35]. Since there is an intracellular pool of APN and APA in glomerular epithelial cells, the results observed in the present study could be attributed to the targeting of a storage cytoplasmic pool of these enzymes to membrane domains. This hypothesis is not likely because PMA for APN as well as cAMP for APA stimulated both cell surface and total cell activities.

The role of APN and APA of glomerular epithelial cells is still unknown. As many ectopeptidases in other tissues, they can regulate autocrine and paracrine signals by proteolytic activation or inactivation of peptidic autacoids. They can also process proproteins into their final active secreted forms. Such effects have been already reported in various preparations. For example, brain and lymphocyte APN were characterized as inactivators of enkephalines [36] and thymopentin [37], respectively. APA transforms by deletion of the N terminal aspartic acid angiotensin II into the less active 2-8 heptapeptide. It may thus regulate the action of angiotensin II in the glomerulus where expression of a local renin-angiotensin system has been demonstrated. APA expression is increased in the glomeruli of the remnant renal tissue of partially nephrectomized rats, which suggests this enzyme may play a role in glomerular adaptations after renal mass ablation [38]. Both APA and APN may also be involved in the final trimming associated with posttranslational modifications of secreted proteins as suggested recently for ectopeptidases in general [39].

The present study demonstrates that the control of APA and APN activities in glomerular epithelial cells is different. This selective expression may provide new insights regarding their possible biological significance. Cell surface peptidases may act as modulators of growth and differentiation. Local increases in glomerular mediators such as thrombin which stimulates PKC or PGE_2 which stimulates cAMP and the protein kinase A pathway are likely to modify aminopeptidase expression. This could represent a supplementary mechanism whereby these autacoids modify the glomerular functions in the course of glomerular diseases.

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